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Phosphorylation of cold shock domain/Y-box proteins by ERK2 and GSK3β and repression of the human VEGF promoter

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Abstract The hypoxia responsive region (HRR) of the VEGF promoter plays a key role in regulating VEGF expression. We found that the cold shock domain (Y-box) repressor proteins, dbpA and dbpB/YB-1, bind distinct strands of the human VEGF HRR. We find both dbpA and dbpB are phosphorylated by ERK2 and GSK3β in vitro, and the binding of dbpB to single-strand VEGF HRR DNA is regulated by this phosphorylation. These findings suggest the ERK/MAPK and PI3K pathways may regulate VEGF expression in part through regulating the action of these repressor proteins.

Keywords: Vascular endothelial growth factor; Cold shock domain protein; Y-box protein; Gene repression

1. Introduction

VEGF is a key regulator of angiogenesis whose expression is important in the development and maintenance of solid tumors. The hypoxia responsive region (HRR) of the VEGF promoter plays a major role in regulating VEGF expression. It is responsive to hypoxia, oncoproteins and growth factors, primarily via activation of function of the positive activator HIF-1 (hypoxia inducible factor 1) through mechanisms targeting the HIF-1α subunit of the HIF-1 complex [1–3]. Signalling to the VEGF HRR can involve both ERK/MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinase)/Akt signalling pathways [1,3,4].

A number of proteins, including VHL, prolyl hydroxylases and the HDM2/p53 pathway, negatively regulate VEGF expression by targeting HIF-1α for degradation when oxygen levels are adequate [5–7]. We recently identified an additional mode of negative regulation of VEGF expression which involves transcriptional repression by members of the cold shock domain (CSD) family of proteins (also known as Y-box proteins) [8]. CSD proteins are primarily single-strand DNA and RNA binding proteins involved in both transcriptional activation and repression, and which also operate at multiple levels in post-transcriptional regulation [9–11]. The two major somatic CSD proteins are dbpB (also known as YB-1, p50, MSY-1, TSEP1, NSEP1) and dbpA (also known as YB-2, ZONAB, MSY3/4). We previously found these proteins contact a site immediately downstream of the HIF-1 binding site on the non-coding strand of the mouse VEGF HRR, and repress transcription from the VEGF HRR in mouse fibroblasts [8].

We now report an analysis of the human VEGF HRR in the MCF-7 human breast cancer cell line. We find that the dbpA and dbpB CSD proteins bind to separate strands of the human HRR, and furthermore that transcriptional repression mediated by these proteins may be regulated by phosphorylation.

2. Materials and methods

2.1. Plasmid constructs

The wild type and mutant human VEGF HRR luciferase reporter constructs were constructed by cloning oligonucleotides shown in Fig. 1 into *Bam*HI/*Xho*I-digested pTK81luc as described previously [8]. Background luciferase activity from pTK81luc does not respond to hypoxia and is not affected by CSD protein overexpression [8,12]. The plasmids pSGdbpA, pSGdbpB, GST-dbpA, GST-dbpB, pSGBdel1 and pSGBdel2 were previously described [8,12]. pGEXBdel1 was generated by cloning *Eco*RI/*Not*I fragments from pSGBdel1 into pGEX4T-1. pGEXAdel1 was created by digesting pGEXA with *Nae*I/*Sma*I to remove the C-terminal and central CSD domains followed by religation. pGEXAdel1 contains the first 77 amino acids of dbpA. dbpA and dbpB GST-peptide fusion constructs, pGEXA-ps and pGEXB-ps contain, respectively, N-terminal amino acids 27–43 and 23–40. PGEXA-psmut and pGEXB-psmut contain ser/thr to ala substitutions in ERK2 and GSK3β phosphorylation sites.

2.2. Preparation of recombinant and nuclear protein

The *Escherichia coli* strain MC1061 transformed with pGEXBT or pGEXA was induced with isopropyl-1-thio-β-D-galactopyranoside to produce recombinant GST-dbpB and -dbpA, respectively. The fusion proteins were purified on glutathione–Sepharose beads as described by the manufacturer (Promega). Crude nuclear extracts from human MCF-7 breast cancer cells were prepared as previously described [8].

2.3. Antibodies

An anti-CSD peptide polyclonal antibody was raised by immunizing rabbits with the peptide (IKKNNPRKYLRSVGD) (human dbpB amino acids 89–103, conserved in dbpA and dbpB) conjugated to keyhole limpet hemocyanin (Imject conjugation kit, Pierce) as previously described [12,8].

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Abbreviations: CSD, cold shock domain; HRR, hypoxia responsive region; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase

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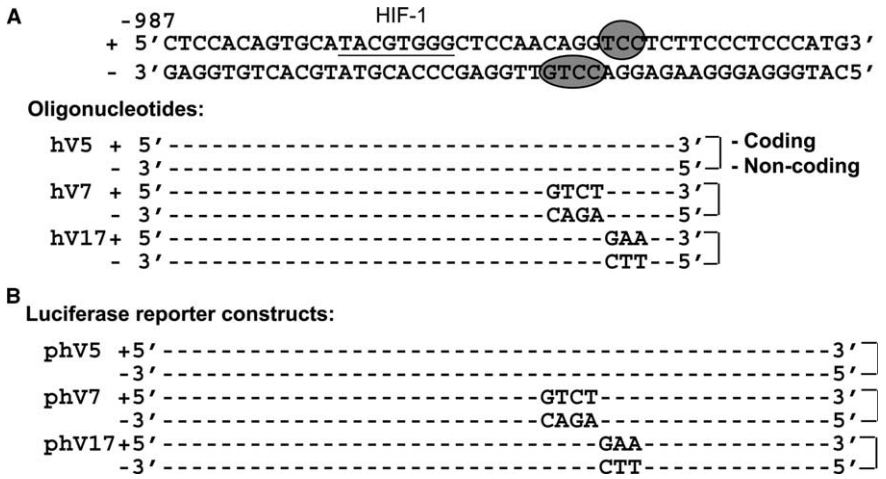

Fig. 1. Sequences of gel shift oligonucleotides and reporter construct inserts from the human VEGF promoter. The sequence of the human VEGF HRR (−987 to −937, corresponding to 1376–1420 of GenBank M63971) is shown with the HIF-1 binding site [13] underlined, and predicted core binding sequences for CSD proteins circled. The coding (+) and non-coding (−) strands are indicated. Bases that vary from the wild type sequence are indicated, with wild type bases shown as dashes.

2.4. Gel shift analysis, competitions, and antibody analysis

Gel shift assays were performed using 0.25 ng of single-strand ^{32}P-labelled oligonucleotide probe in a 10 μl reaction mix of 0.5× TM buffer containing 200 mM KCl, 0.4 μg of poly(dI-dC) and either 1 μg of crude nuclear extract or 25 ng of recombinant CSD fusion protein (GST-dbpB, GST-dbpA), as described [9,8]. Gel shift assays using recombinant protein also contained 2 μg of bovine serum albumin. Reactions were incubated at room temperature for 20 min and analyzed on 12% or 6% non-denaturing polyacrylamide gels for analysis of nuclear extracts and recombinant protein, respectively. Competition with unlabelled single-strand oligonucleotides was performed by addition of protein and 5 ng of unlabelled probe, followed by immediate addition of the ^{32}P-labelled probe. Antibody blocking experiments were performed by adding protein and antibody and incubating for 5 min at room temperature before adding the ^{32}P-labelled probe. The reaction was incubated for an additional 20 min at room temperature before being analyzed on polyacrylamide gels. Oligonucleotides used in retardation assays are shown in Fig. 1.

2.5. Analysis of phosphorylated recombinant CSD proteins

Recombinant GST-CSD fusion proteins (dbpA, dbpB/YB-1; 50 ng) were assayed for phosphorylation by GSK3β or ERK2 using [γ-^{32}P]ATP in an in vitro phosphorylation assay, as described by the supplier (New England Biolab). Kinase reactions were examined by SDS/PAGE and ^{32}P-labelled proteins detected after exposure of gels to X-ray film. Proteins were sized relative to marker proteins. For preparation of cold-kinased proteins, kinase reactions were performed in the presence of 0.5 mM ATP in the absence or presence of GSK3β or ERK2. Cold kinased or mock kinased proteins were then immediately analyzed in a gel shift reaction as described above.

2.6. Cell culture and transfection

MCF-7 breast cancer cells were grown in Dulbecco's modified Eagle's medium and 10% fetal calf serum. 2×10^5 cells were transfected with 500 ng of luciferase reporter constructs or cotransfected with 500 ng reporter constructs and 250 ng of dbpB (pSGdbpB) or dbpA (pSGdbpA) overexpression constructs. Transfections were performed using Lipofectamine™ 2000 (Gibco BRL) according to the manufacturers directions. 24 hours after transfection, cells were either placed in hypoxic conditions (1% O_2) or left in normoxic conditions (20% O_2) for 16 h. For treatment with kinase pathway inhibitors, 50 μM PD98059 or LY294002 (Promega) in DMSO or DMSO alone (final concentration 0.05%) were added 30 min prior to treatment. Hypoxic conditions were generated in a hypoxic chamber (Edwards Instrument Company, Sydney, Australia). Luciferase activity was determined as previously described [8].

3. Results

3.1. Strand-specific binding of dbpA and dbpB/YB-1 to the human VEGF HR region

Having found previously that CSD proteins repress mouse VEGF transcription by binding to single-stranded elements in the HRR [8], we wished to check that the human VEGF gene is subject to similar regulation. First we assessed the binding of recombinant GST-dbpA and GST-dbpB/YB-1 to individual strands of the human VEGF HRR in gel shift assays. GST-dbpA preferentially bound the HRR lower strand probe (i.e., the non-coding strand, V5−), whereas GST-dbpB preferentially bound the coding strand (V5+) (Fig. 2A).

To examine whether CSD proteins in nuclear extracts from MCF-7 human breast cancer cells can participate in complex formation on the individual human HRR strands, we performed gel shift assays in the presence or absence of an inhibitory anti-CSD antibody [8,12] or preimmune serum. The major complexes that formed on each strand were distinct, but their respective formations were strongly inhibited by the CSD antibody, but not preimmune serum, suggesting that the major complexes forming on each strand contain CSD proteins (Fig. 2B). To indicate whether dbpB participates in formation of either of the complexes, we added a competitor oligonucleotide that has previously been demonstrated to bind dbpB [9]. This oligonucleotide (GM−), which is derived from the promoter of the GM-CSF gene, competed for complex formation on the V5+ probe, but not on the V5− probe (Fig. 2C), indicating that dbpB binds the HRR coding strand, but not the non-coding strand, consistent with the binding preference observed for recombinant dbpB (Fig. 2A).

Based on previous findings of binding site preferences for CSD proteins [8,9] we introduced point mutations into the HRR coding and non-coding strand probes and examined the effect on binding of recombinant CSD proteins or MCF-7 nuclear extracts. Replacement of 5′CCTG3′ in the non-coding strand with 5′AGAC3′ (probe V7−) reduced the binding of recombinant GST-dbpA, and correlating with this also reduced the formation of the upper band of the two bands that

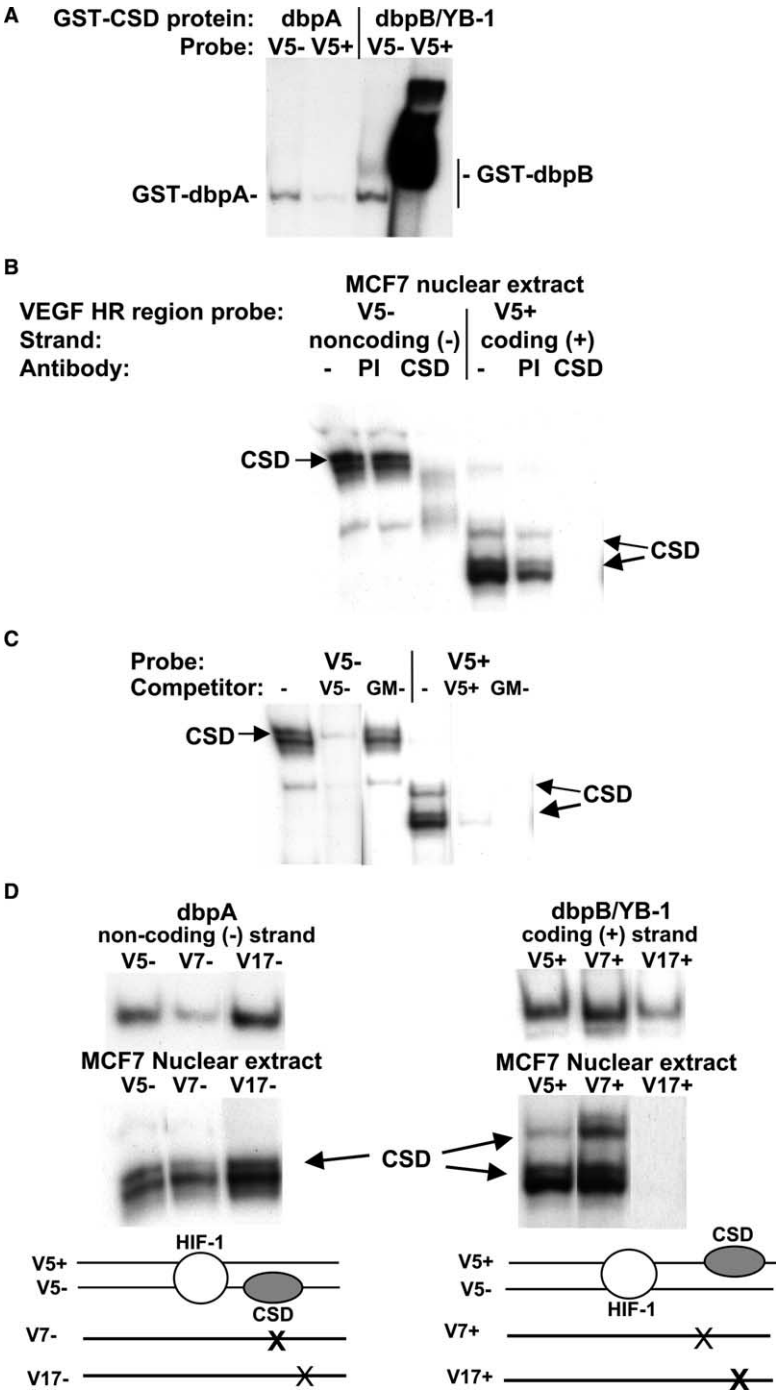

Fig. 2. Strand-specific binding of dbpA and dbpB CSD proteins to the human VEGF HRR (A) Gel shift assays of GST-dbpA and GST-dbpB with ^{32}P-labelled VEGF HRR coding (V5+) and non-coding (V5−) single-strand DNA probes. (B) Gel shift assays of nuclear extracts from MCF-7 cells that had been preincubated with preimmune serum (PI), with a pan-specific anti-CSD antibody (CSD) or left untreated (−), binding to ^{32}P-labelled VEGF HRR coding (V5+) and non-coding (V5−) single-strand DNA probes. (C) Gel shift competition assays of MCF-7 nuclear extracts that had been preincubated with unlabelled VEGF HRR coding stand (V5+), VEGF HRR non-coding strand (V5−) or an oligonucleotide from the GM-CSF gene (GM-) that binds dbpB [9]. (D) Gel shift assays of GST-dbpA or GST-dbpB or MCF-7 nuclear extracts binding to ^{32}P-labelled wild type (V5) and mutant (V7,V17) VEGF HRR non-coding (−) and coding (+) strand DNA probes. A schematic of the VEGF HRR showing HIF-1 and predicted CSD protein binding sites is given below, with mutated regions indicated with an X.

form on the coding strand probe in the presence of nuclear extract (Fig. 2D). Replacement of 5′TTC3′ in the upper strand probe with 5′GAA3′ (probe V17+) reduced the binding of recombinant dbpB and eliminated the formation of complexes from nuclear extract (Fig. 2D). Together these data indicate that both recombinant and nuclear dbpA binds the upper strand of the HRR in vitro, and recombinant and nuclear dbpB binds the lower strand.

3.2. Repressive role of dbpA and dbpB/YB-1 and the MAPK pathway in normoxic MCF-7 cells

To test the function of the dbpA and dbpB binding sites in the human VEGF HRR, the HRR was inserted upstream of the minimal thymidine kinase promoter of a luciferase reporter gene and the effect of mutation of the dbpA or dbpB binding sites on luciferase expression in transfected MCF-7 cells was determined. Mutation of either site resulted in increased expression under normoxia (Fig. 3), consistent with our previous report that CSD proteins act as repressors of VEGF transcription in mouse fibroblasts [8]. The effect of CSD protein binding in hypoxia could not be tested by mutation of the sites, because they overlap the site for a positively acting hypoxia ancillary factor [13]. To test whether dbpA and dbpB can each act as repressors, expression vectors for each were cotransfected with the VEGF HRR-luc reporter gene, hV5luc. Coexpression of dbpA reduced luciferase activity by 60–63%, while coexpression of dbpB reduced expression by 45% in normoxia, and by 67% in hypoxia (Fig. 3B), consistent with both proteins being able to repress transcription from the VEGF HRR.

Because the ERK/MAPK and PI3K pathways have both been found to influence VEGF expression [1,3,14], we tested whether these pathways influence the expression of luciferase from the VEGF HRR-luc reporter. Addition of the ERK pathway inhibitor PD98059 for 16 h prior to harvest resulted in 2.6-fold and 2.2-fold increases in luciferase expression in normoxia and during hypoxia (Fig. 3C). This suggests that the ERK pathway, even in unstimulated cells, maintains the activity of a repressor of the VEGF HRR. Treatment with the PI3K pathway inhibitor, LY294002, had little effect on luciferase expression in normoxic cells, but caused a substantial reduction in expression in hypoxic cells (Fig. 3C). This indicates that activation of the PI3K pathway contributes to transcription from the VEGF HRR in hypoxic MCF-7 cells.

3.3. CSD protein phosphorylation by ERK2 and GSK3β and enhanced binding of dbpB to the VEGF HR region

Given the repressive action of the ERK pathway on expression from the HRR, we wished to assess whether the dbpA or dbpB repressor proteins might be targets for ERK pathway kinases. We noticed that both dbpA and dbpB contain potential phosphorylation sites [15,16] for ERK1/2 in their N-terminal regions, and that the potential ERK sites in each protein had adjacent consensus sequences for phosphorylation by GSK3β (Fig. 4), a kinase that is negatively regulated by the PI3K pathway [17]. To indicate whether these putative phosphorylation sites might be targets for ERK and/or GSK3β, we tested the ability of the kinases to phosphorylate recombinant dbpA and dbpB in vitro. Both GST-dbpA and GST-dbpB, but not

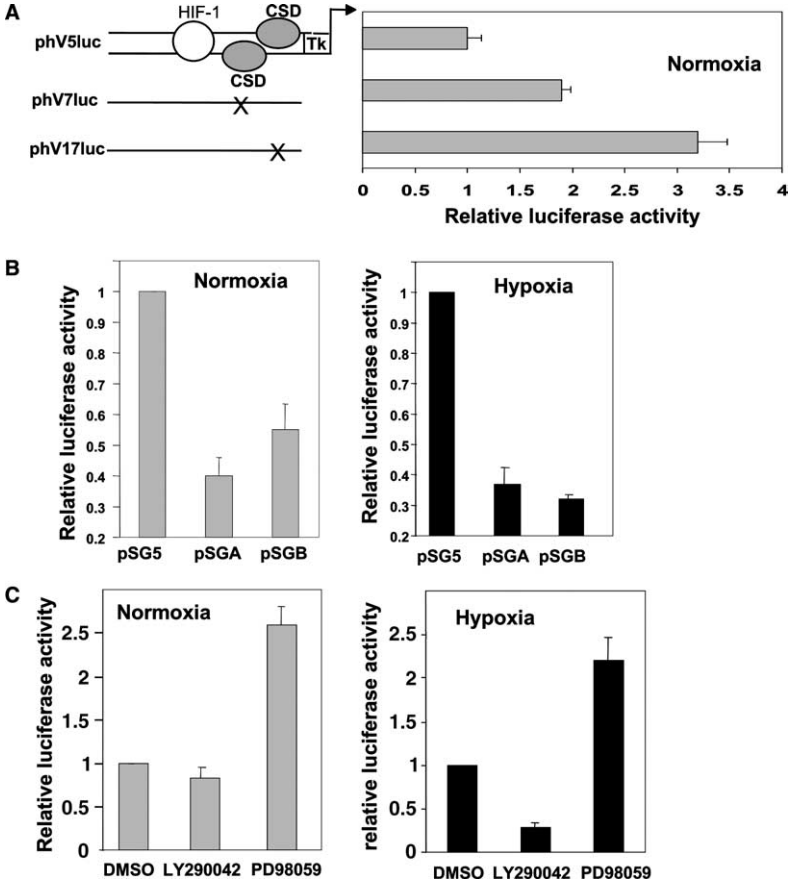

Fig. 3. Role of CSD binding sites and signalling pathways in repression of the human VEGF HRR in MCF-7 cells. (A) Luciferase activity from wild type (phV5luc) and mutant VEGF HRR reporter constructs. (B) Effect of cotransfection of dbpA (pSGA), dbpB (pSGB) or empty vector (pSG5) on expression from the VEGF HRR. (C) Effect of the PI3K pathway inhibitor LY294002, or the MAPK pathway inhibitor PD98059, on expression from the VEGF HRR. The inhibitors did not affect expression from the backbone luciferase vector pTK81luc, and DMSO treatment did not affect expression from phV5luc (data not shown).

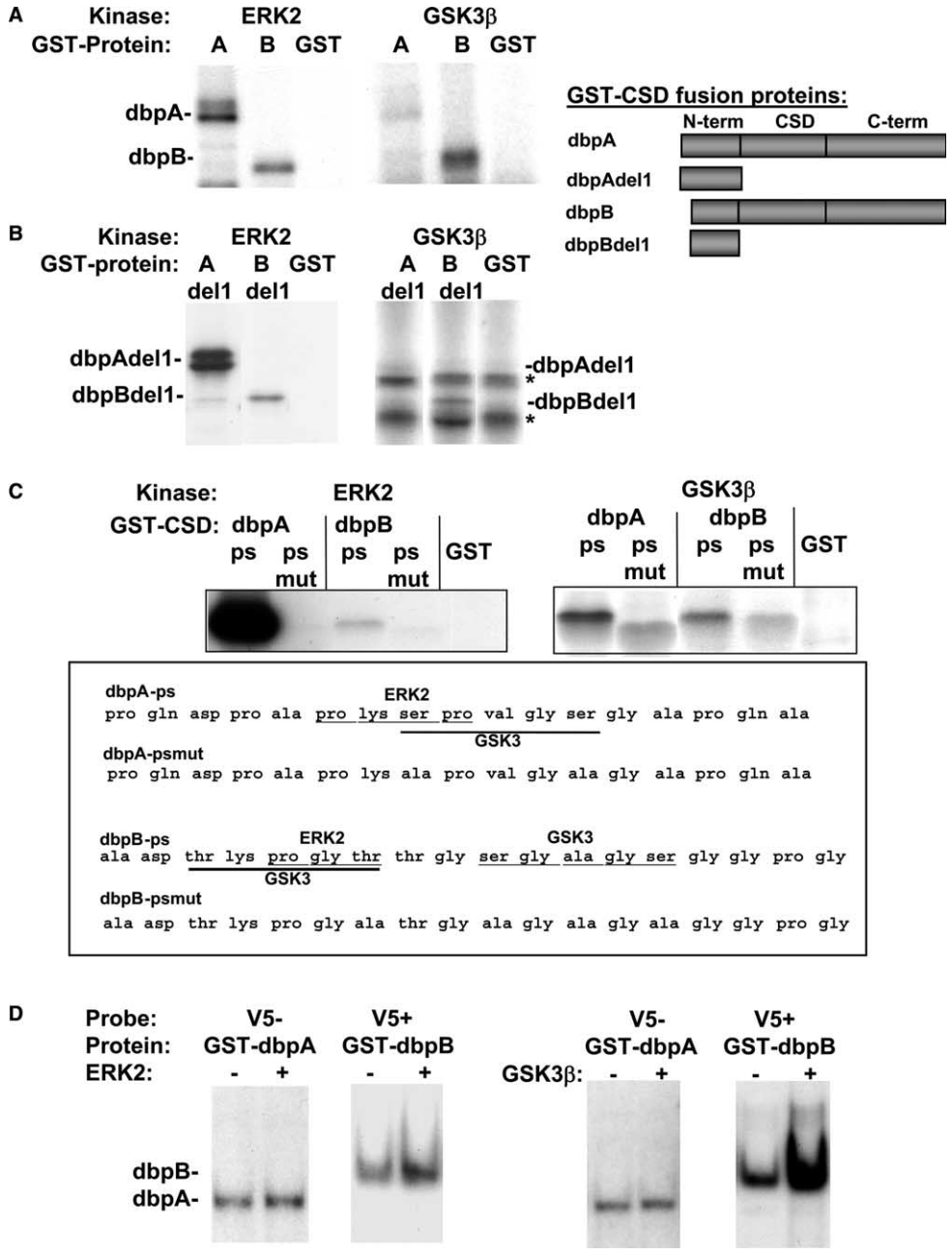

Fig. 4. Phosphorylation of dbpA and dbpB by ERK2 and GSK3β . (A) GST-dbpA and dbpB were phosphorylated in vitro using ERK2 or GSK3β and electrophoresis on SDS/PAGE. (B) Phosphorylation of the N-terminal domains of dbpA and dbpB by ERK2 and GSK3β. Auto-phosphorylated proteins, present in GSK3β assays without any added recombinant protein, are indicated by asterisks. (C) Recombinant GST-peptide (ps) and phosphorylation site mutant peptides (psmut) were assayed for phosphorylation by ERK2 and GSK3β. The N-terminal dbpA and dbpB sequences contained in fusion proteins are shown, with ERK2 and GSK3β sites underlined. (D) Gel shift assays of GST-dbpA and GST-dbpB binding to their respective strands of the HRR, with and without prior phosphorylation by ERK2 and GSK3β with unlabelled ATP.

GST alone, were readily phosphorylated in vitro by purified ERK2 (Fig. 4A), as was GST fusion protein containing just the N-terminal domain of dbpA (dbpAdel1) or dbpB (dbpBdel1) (Fig. 4B). GST-dbpB was also readily phosphorylated in vitro by purified GSK3β, while GST-dbpA was phosphorylated to a lesser extent (Fig. 4A). Again, the fusion proteins containing just the N-terminal domains were also phosphorylated (Fig. 4B). To test whether the predicted sites in the N-terminal domains were sites of phosphorylation, we constructed GST fusion proteins, dbpA-ps and dbpB-ps, containing short peptides from the dbpA and dbpB N-terminal domains that included the predicted phosphorylation sites, as well as mutant forms (dbpA-psmut, dbpB-psmut) in which the serine or threonine residues at the predicted phosphorylation sites were changed to alanine (Fig. 4C). The fusion proteins containing the wild type peptide regions were phosphorylated by ERK2 and GSK3β in vitro, but phosphorylation was reduced or abolished by mutation of the predicted ERK2 and GSK3β sites (Fig. 4C).

Finally, we tested whether phosphorylation of dbpA or dbpB affected their binding to the VEGF HRR in vitro. GST-dbpA and GST-dbpB fusion proteins were phosphory-

lated in vitro by ERK2 or GSK3β and tested for binding to VEGF HRR probes in a gel shift assay. Phosphorylation by both ERK2 and GSK3β increased the binding of dbpB to the VEGF HRR coding strand, but did not appreciably affect the binding of dbpA to the non-coding strand (Fig. 4D). Binding of each protein to the opposite strands remained negligible (data not shown). Phosphorylation of dbpB may therefore regulate its binding to the VEGF HRR. The increase in DNA binding by ERK2 phosphorylation is consistent with our finding that the ERK pathway inhibitor, PD98059, increases expression from the VEGF HRR, while the increase in DNA binding by GSK3β is consistent with its proposed involvement in repression of VEGF HRR activity [4,18].

4. Discussion

The binding of CSD proteins to single-strand DNA is thought to play a major role in repression by these proteins and is proposed to block the binding of activator factors that require double-strand DNA [8,9,19–21]. Sequence-specific single-strand DNA binding is directed by a central conserved 70 amino acid CSD domain [22] but can be modulated by the flanking N-terminal sequences [23,24]. The binding of CSD protein complexes to both strands of the human VEGF HRR may secure the region in a stable single-stranded repressive structure that prevents inappropriate activation of this region under unstimulated conditions.

We found that CSD proteins are targets for signalling pathways that regulate the activity of the VEGF HRR in MCF-7 cells. We demonstrate for the first time that dbpA and dbpB/YB-1 can be phosphorylated by ERK2, which is positively regulated by the MAPK pathway [15], and also by GSK3β which is negatively regulated by the PI3K pathway [25]. GSK3β is constitutively active in unstimulated cells and is inactivated in hypoxic conditions by PI3K pathway-activated Akt [25]. Conceivably, a reduction in CSD protein phosphorylation on activation of the PI3K/Akt pathway could augment the effect this pathway has in some cell types in increasing HIF-1 activity during hypoxia [26] or in response to growth factors [2]. We found that the binding of dbpB/YB-1 to the VEGF HR region coding (+) strand DNA was enhanced by both GSK3β and ERK2 phosphorylation and the binding of dbpA to the non-coding (−) strand was slightly enhanced by ERK2 phosphorylation. GSK3β and ERK2 may therefore play a role in increasing CSD protein binding to the VEGF promoter and may enhance the repressive ability of these proteins, although further work is required to verify that the CSD proteins are phosphorylated in vivo by ERK and GSK3. Although phosphorylation of dbpA had little effect on DNA binding of the recombinant protein, it cannot be ruled out that phosphorylation plays a role in nuclear dbpA complex formation. We observed that recombinant dbpA binds poorly compared to the CSD protein-containing nuclear complex that forms on the non-coding strand, and it is possible that phosphorylation may contribute to this difference. Phosphorylation may also be involved in cross talk between the CSD complexes binding to the two VEGF HRR strands, to assist in stabilizing a repressive single-strand structure.

An interesting parallel to the mode of action of dbpB in repressing the VEGF gene, and the potential regulation by phosphorylation, has been described in a report on the regulation of thyrotropin gene transcription by the rat dbpB/YB-1 homologue, TSEP-1 [27]. TSEP-1 binds to single-stranded motifs in the thyrotropin gene promoter and is a repressor of thyrotropin receptor gene transcription. In addition, the binding of TSEP-1 in vitro was found to be enhanced by prior in vitro phosphorylation by protein kinase A. YB-1 has also been shown to be phosphorylated in vitro by casein kinase II [28], which may participate in regulating its nuclear translocation [29].

We found that the MAPK pathway in MCF-7 cells represses expression from the VEGF HRR, in apparent contrast to a number of reports that show the MAPK pathway activates expression from HRE-containing reporter genes [30–32,18]. In some cases the difference may simply be due to the use in the reporter of the erythropoietin HRE [30–32], which does not contain obvious sites for binding CSD proteins. Nevertheless, it is established in a number of cell types that activation of the MAPK pathway leads to increased HIF-1α transactivating activity [30–32,18], although the degree to which the MAPK pathway affects HIF-1α is known to vary between different cell lines [3]. It is possible that the overall outcome in each cell type depends on the balance of contributions from the opposing effects of enhanced repression by CSD proteins on the one hand and enhanced transactivating activity of HIF-1 on the other. The only other report we are aware of that indicates repression of VEGF expression by a MAPK pathway is the finding that the ERK5 MAPK acts as a repressor of VEGF in mouse embryonic fibroblasts [33]. It would be interesting to determine whether the CSD proteins are substrates for ERK5. The effect of the MAPK pathway we observed in normoxia is consistent with reports demonstrating the presence of activated/phosphorylated ERK1/2 in both stimulated and unstimulated MCF-7 cells [34].

We localized phosphorylation sites for GSK3β and ERK2 to the N-terminal regions of dbpA and dbpB/YB-1. Examination of the protein sequences revealed potential ERK2 [25,35] and GSK3β [15,16] phosphorylation sites within the N-terminal regions of dbpA and dbpB/YB-1, but no apparent sites in the CSD or C-terminal domains. Mutation of the predicted sites confirmed that they are targets for the two kinases. Consistent with the observed effect of CSD protein phosphorylation on DNA binding (primarily on dbpB/YB-1), CSD protein N-terminal regions have been shown to affect the efficiency of CSD protein binding to single-strand DNA [36,23]. Taken all together, our data suggest that in normoxic conditions the binding of dbpA and dbpB/YB-1 to each strand of the VEGF HR region results in efficient repression, presumably by the creation of a stable single-strand structure which would inhibit the binding of activators that require double-strand DNA. The CSD proteins would therefore function to maintain the HR region in a repressed state in the absence of activating stimuli. Phosphorylation by ERK and GSK3β in normoxic cells would contribute to the stabilization of CSD proteins binding to single strand DNA, in particular to the binding by dbpB/YB-1. Under hypoxic conditions CSD protein binding may be reduced by the loss of GSK3β phosphorylation, allowing displacement of CSD proteins and the binding of double-strand DNA-dependent hypoxic activators such as HIF.

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References

[1] Berra, E., Pages, G. and Pouyssegur, J. (2000) MAP kinases and hypoxia in the control of VEGF expression. Cancer Metastasis Rev. 19, 139–145.
[2] Laughner, E., Taghavi, P., Chiles, K., Mahon, P.C. and Semenza, G.L. (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell Biol. 21, 3995–4004.
[3] Semenza, G.L. (2002) Signal transduction to hypoxia-inducible factor 1. Biochem. Pharmacol. 64, 993–998.
[4] Seta, K.A., Spicer, Z., Yuan, Y., Lu, G. and Millhorn, D.E. (2002) Responding to hypoxia: lessons from a model cell line. Science stke 146/re11, 1–16.
[5] Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin Jr., W.G. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292, 464–468.
[6] Baek, J.H., Mahon, P.C., Oh, J., Kelly, B., Krishnamachary, B., Pearson, M., Chan, D.A., Giaccia, A.J. and Semenza, G.L. (2005) OS-9 interacts with hypoxia-inducible factor 1alpha and prolyl hydroxylases to promote oxygen-dependent degradation of HIF-1alpha. Mol. Cell 17, 503–512.
[7] Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L. and Bedi, A. (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. 14, 34–44.
[8] Coles, L.S., Diamond, P., Lambrusco, L., Hunter, J., Burrows, J., Vadas, M.A. and Goodall, G.J. (2002) A novel mechanism of repression of the vascular endothelial growth factor promoter, by single strand DNA binding cold shock domain (Y-box) proteins in normoxic fibroblasts. Nucleic Acids Res. 30, 4845–4854.
[9] Coles, L.S., Diamond, P., Occhiodoro, F., Vadas, M.A. and Shannon, M.F. (2000) An ordered array of cold shock domain repressor elements across tumor necrosis factor-responsive elements of the granulocyte–macrophage colony-stimulating factor promoter. J. Biol. Chem. 275, 14482–14493.
[10] Matsumoto, K. and Wolffe, A.P. (1998) Gene regulation by Y-box proteins: coupling control of transcription and translation. Trends Cell Biol. 8, 318–323.
[11] Kohno, K., Izuma, H., Uchiumi, T., Ashizuka, M. and Kuwano, M. (2003) The pleiotropic functions of the Y-box-binding protein, YB-1. Bioessays 25, 691–698.
[12] Diamond, P., Shannon, M.F., Vadas, M.A. and Coles, L.S. (2001) Cold shock domain factors activate the granulocyte–macrophage colony-stimulating factor promoter in stimulated Jurkat T cells. J. Biol. Chem. 276, 7943–7951.
[13] Kimura, H., Weisz, A., Ogura, T., Hitomi, Y., Kurashima, Y., Hashimoto, K., D'Acquisto, F., Makuuchi, M. and Esumi, H. (2001) Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. J. Biol. Chem. 276, 2292–2298.
[14] Michiels, C., Minet, E., Michel, G., Mottet, D., Piret, J.-P. and Raes, M. (2001) HIF-1 and AP-1 cooperate to increase gene expression in hypoxia: role of MAP kinases. IUBMB Life 52, 49–53.
[15] Davis, R.J. (1993) The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 268, 14553–14556.
[16] Pouyssegur, J. and Lenormand, P. (2003) Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. Eur. J. Biochem. 270, 3291–3299.
[17] Dajani, R., Fraser, E., Roe, S.M., Young, N., Good, V., Dale, T.C. and Pearl, L.H. (2001) Crystal structure of glycogen synthase kinase 3β: structural basis for phosphate-primed substrate specificity and autoinhibition. Cell 105, 721–723.
[18] Sodhi, A., Montaner, S., Miyazaki, H. and Gutkind, J.S. (2001) MAPK and Akt act cooperatively but independently on hypoxia inducible factor-1α in *ras*V12 upregulation of VEGF. Biochem. Biophys. Res. Commun. 287, 292–300.
[19] MacDonald, G.H., Itoh-Lindstrom, Y. and Ting, J.P. (1995) The transcriptional regulatory protein, YB-1, promotes single-stranded regions in the DRA promoter. J. Biol. Chem. 270, 3527–3533.
[20] Horwitz, E.M., Maloney, K.A. and Ley, T.J. (1994) A human protein containing a "cold shock" domain binds specifically to H-DNA upstream from the human gamma-globin genes. J. Biol. Chem. 269, 14130–14139.
[21] Norman, J.T., Lindahl, G.E., Shakib, K., En-Nia, A., Yilmaz, E. and Mertens, P.R. (2001) The Y-box binding protein YB-1 suppresses collagen alpha 1(I) gene transcription via an evolutionarily conserved regulatory element in the proximal promoter. J. Biol. Chem. 276, 29880–29890.
[22] Graumann, P.L. and Marahiel, M.A. (1998) A superfamily of proteins that contain the cold-shock domain. Trends Biochem. Sci. 23, 286–290.
[23] Kolluri, R., Torrey, T.A. and Kinniburgh, A.J. (1992) A CT promoter element binding protein: definition of a double-strand and a novel single-strand DNA binding motif. Nucleic Acids Res. 20, 111–116.
[24] Nambier, A., Swamynathan, S.K., Kandala, J.C. and Guntaka, R.V. (1998) Characterization of the DNA-binding domain of the avian Y-box protein, chkYB-2, and mutational analysis of its single-strand binding motif in the Rous sarcoma virus enhancer. J. Virol. 72, 900–909.
[25] Harwood, A.J. (2001) Regulation of GSK-3: a cellular multiprocessor. Cell 105, 821–824.
[26] Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A.R., Ryan, H.E., Johnson, R.S., Jefferson, A.B., Stokoe, D. and Giaccia, A.J. (2000) Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev. 14, 391–396.
[27] Ohmori, M., Shimura, H., Shimura, Y. and Kohn, L.D. (1996) A Y-box protein is a suppressor factor that decreases thyrotropin receptor gene expression. Mol. Endocrinol. 10, 76–89.
[28] Skabkin, M.A., Evdokimova, V., Thomas, A.A. and Ovchinnikov, L.P. (2001) The major messenger ribonucleoprotein particle protein p50 (YB-1) promotes nucleic acid strand annealing. J. Biol. Chem. 276, 44841–44847.
[29] Higashi, K., Inagaki, Y., Fujimori, K., Nakao, A., Kaneko, H. and Nakatsuka, I. (2003) Interferon-γ interferes with transforming growth factor-β signaling through direct interaction of YB-1 with SMAD3. J. Biol. Chem. 278, 43470–43479.
[30] Minet, E., Arnould, T., Michel, G., Roland, I., Mottet, D., Raes, M., Remacle, J. and Michiels, C. (2000) ERK activation upon hypoxia: involvement in HIF-1 activation. FEBS Lett. 468, 53–58.
[31] Mottet, D., Michel, G., Renard, P., Ninane, N., Raes, M. and Michiels, C. (2003) Role of ERK and calcium in the hypoxia-induced activation of HIF-1. J. Cell Physiol. 194, 30–44.
[32] Hur, E., Chang, K.Y., Lee, E., Lee, S.K. and Park, H. (2001) Mitogen-activated protein kinase kinase inhibitor PD98059 blocks the trans-activation but not the stabilization or DNA binding ability of hypoxia-inducible factor-1alpha. Mol. Pharmacol. 59, 1216–1224.
[33] Sohn, S.J., Sarvis, B.K., Cado, D. and Winoto, A. (2002) ERK5 MAPK regulates embryonic angiogenesis and acts as a hypoxia-sensitive repressor of vascular endothelial growth factor expression. J. Biol. Chem. 277, 43344–43351.
[34] Tsai, P.-W., Shiah, S.G., Lin, M.-T., Wu, C.-W. and Kuo, M.-L. (2003) Up-regulation of vascular endothelial growth factor C in breast cancer cells by heregulin-β1. J. Biol. Chem. 278, 5750–5759.
[35] Frame, S., Cohen, P. and Biondi, R.M.. (2001) A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. Mol. Cell 7, 1321–1327.
[36] Shannon, M.F., Coles, L.S., Attema, J. and Diamond, P. (2001) The role of architectural transcription factors in cytokine gene transcription. J. Leukoc. Biol. 69, 21–32.